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14. ABSTRACT A major clinical manifestation of breast cancer patients is the development of bone metastasis. Several lines of experimental evidence suggested that chemokine receptor CXCR4 and its ligand SDF-1α signaling molecules may play an important role in the bone metastasis of breast cancer via activating NF-κB, a key transcription factor. We have previously shown that Indole-3-carbinol (I3C), a natural compound present in vegetables of the genus Brassica can inhibit NF-κB in breast cancer cells. Therefore, we hypothesize that I3C may be able to inhibit the bone metastasis of breast cancer by inhibition of NF-κB, resulting in inhibition of CXCR4/SDF-1α and other NF-κB targeted genes. This is the first time report that we have established an animal model of breast cancer bone metastasis using SCID human (SCID-Hu) animal. Using this model, we investigated the effect of I3C on MDA-MB-231 breast cancer cells and experimental MDA-MB-231 bone tumors created by injecting MDA-MB-231 cells into human bone fragments. We found that I3C significantly inhibited MDA-MB-231 bone tumor growth. This correlated well with down regulation of NF-κB. We studied the mRNA expression of CXCR4 and SDF-1α and found that 60μM I3C significantly inhibited SDF-1α and CXCR4 expression in MDA-MB-231 cells. Our hypotheses will be tested using MDA-MB-231 human breast cancer cells growing in the SCID-Hu model, wherein we can document the expression profile of specific targeted genes could be altered by I3C treatment. By using a novel siRNA technology to silence the SDF-1α and/or CXCR4 genes, we plan to study how SDF-1α/CXCR4 signaling affects the infiltrating growth of MDA-MB-231 cells to the bone in the SCID-Hu model, and to delineate whether the effects are exerted via activation of MMPs, uPA and NF-κB (1). The data obtained from our experiments will provide novel pathways that could be exploited for the prevention and/or treatment of breast cancer bone metastasis in the clinic. Based on above hypothesis, this proposal seems highly relevant to the mission of the Department of Defense.

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INTRODUCTION

The majority of patients dying from cancer of the breast have metastasis to the bone or visceral site (1). The present options for treating breast cancer bone metastasis are limited. Therefore, studies to elucidate molecular mechanism(s) of breast cancer bone metastasis and its prevention is of paramount importance. Several lines of experimental evidences have suggested that chemokine receptor CXCR4 and its ligand SDF1-α are over-expressed in metastatic breast cancer cells (2). Moreover, NF-κB, a key transcription factor may regulate the expression and function of CXCR4 and/or SDF-1a and activate other metastasis-promoting molecules (uPA, MMPs) in breast cancer cells (3, 4). Thus, agents that directly block the expression of CXCR-4 and/or SDF-1α signaling partly and due to inhibition of NF-κB may have great therapeutic potential for treating metastatic breast cancer. It has been shown that Indole-3-carbinol (I3C) can inhibit NF-κB in breast cancer cells (5). Recently, we studied the mRNA expression of CXCR4 and SDF-1α and found that I3C, significantly inhibited CXCR4/SDF-1α expression in breast cancer cells. However little is known about how chemopreventive agents inhibits these metastasis-promoting molecules and therefore, we hypothesize that I3C may be able to inhibit the bone metastasis of breast cancer by inhibition of NF-κB and thereby inhibiting CXCR4/SDF-1α, and other NF-κB targeted genes. However, there is no suitable model to investigate the molecular biology of breast cancer metastasis and we trust that SCID-Hu model could be a suitable model to fill this void in our understanding of breast cancer bone metastasis. In this study, we have utilized our recently established the SCID-Hu model of breast cancer bone metastasis to determine the effect of I3C *in vivo.* The purpose of our current investigation: is 1) to determine the effects of I3C on breast cancer cells *in vitro*; 2) to determine the effects of I3C on breast cancer bone tumor growth *in viv*o; 3) to determine the alterations in gene expressions by I3C treatment in both *in vitro* and *in vivo* studies; 4) to compare the gene expression of MDA-MB-231 bone tumors and MDA-MB-231 subcutaneous tumors in SCID mice so as to better understand the molecular mechanism(s) by which I3C exerts its anti-metastatic effect on breast cancer cells; and 5) to determine whether down-regulation of CXCR4 and /or SDF-1α using siRNA approach will prevent the bone metastasis of MDA-MB-231 cells to the bone in the SCID-Hu model.

BODY OF REPORT

The original statement of work in the proposal is listed below:

Task 1: To determine whether metastasis of breast cancer cells to the bone requires both expression of SDF-1α and CXCR4.

a. We will determine whether BT-20 and MDA-MB-231 breast cancer cells can establish bone metastatic tumor and show infiltrating growth in the SCID-Hu model but not in the subcutaneous site of the same SCID mouse. We will determine the expression patterns of SDF-1α and CXCR4 in different cellular compartment (Months 1-3).

b. We will study whether down-regulation of CXCR4 and/or SDF-1α using siRNA approach will prevent the bone metastasis of BT-20 and MDA-MB-231 cells to the bone in the SCID-Hu model (Months 1-3).

c. To explore whether SDF-1α, CXCR4, MMP-9, MMP-2, IL-8, uPA, and NF-κB are involved in the differential metastasis and infiltrating growth of BT-20 and MDA-MB-231 cells in the SCID-Hu environment and in the subcutaneous site (Months 4-6).

Task 2: To determine whether I3C can inhibit the bone metastasis of BT-20 and MDA-MB-231 breast cancer cells and whether the mechanism(s) for the possible inhibition is mediated by down-regulation of SDF-1α, CXCR4, MMPs, IL-8, uPA and NF-κB.

a. We will determine whether systematic treatment of the host mice with I3C will prevent the bone metastasis of BT-20 and MDA-MB-231 cells in the SCID-Hu model (Months 7-10).

b. We will determine whether the possible inhibition of bone metastasis of BT-20 and MDA-MB-231 cells in the SCID-Hu model is associated with down regulation of the expression of CXCR4 in the metastatic tumor cells and SDF-1α, MMP-2, MMP-9, IL-8, uPA and NF-κB in the bone marrow stromal cells (Months 10-12).

c. We will summarize all the data, complete writing of all manuscripts, and submit the final report to DOD (Month 12).

Our progress was slow and we got an extension for one year with no cost. However we are providing the evidences to support the research accomplishments we made last year and indicated that we have completed majority parts of task1 and 2 and plan to complete the rest of task in the extended year. We already reported our present data during the Era of Hope meeting, June 8-11, 2005. It is important to note that BT-20 cell did not grow in the bone. Therefore we did not perform any experiment using BT-20 cell line. In our system MDA-MB-231 gave us good data. Therefore to complete task 1 and task 2, we will stay with MDA-MB-231. Once the tasks 1 and 2 are all completed, we will make progress on data analysis and manuscript writing during the extended year.

We are now reporting the research accomplishments associated with task 1 and 2 outlined in the Statement of Work.

Task 1: To determine whether metastasis of breast cancer cells to the bone requires both expression of SDF-1 α and CXCR4.

MATERIAL AND METHODS:

Animal care and human bone implantation

Female homozygous CB-17 scid/scid mice, aged 4 weeks, were purchased from Taconic Farms (Germantown, NY). The mice were maintained according to the National Institutes of Health standards established in the "Guidelines for the Care and Use of Experimental Animals," and all experimental protocols were approved by the Animal Investigation Committee of Wayne State University (Detroit, MI). Human fetal bone tissue was obtained by a third-party, nonprofit organization (Advanced Bioscience Resources, Alameda, CA), and written informed consent was obtained from the donor, consistent with regulations issued by each state involved and the federal government. Isoflurane anesthesia was used during all surgical procedures. After 1 week of acclimatization, the mice were implanted with a single human fetal bone fragment as described previously (1).

Production of breast cancer bone tumors and I3C treatment

Suspensions of MDA-MB-231 cells ($2x10^5$ cells in a volume of 20 ml of PBS) were injected using a 27-gauge needle through the mouse skin directly into the marrow of implanted fetal bone. The mice were then divided into two groups: Control (n = 10) and Intervention (n = 10) groups (Fig 2). Sesame seed oil was used to facilitate gavage and avoid irritation of the esophagus and was safe as shown also by others (2, 3). The mice in the intervention group were given I3C (1 mg/day/mouse) by oral gavage as soon as the majority of the bone implants began to enlarge (now called a "bone tumor") as determined by caliper measurements (23rd day after cancer cell injection). The control mice received only saline without I3C. The volume of the bone tumor in each group was determined by twice-weekly caliper measurements according to the formula $ab^2 / 2$, where a = length and b = shortest measurement. Percent (%) reduction in tumor volume at the end of the treatment was deduced by the formula: volume of tumor in experimental group ÷ volume in control mice, x 100. The statistical significance of differential findings between the experimental groups and control was determined by student *t*-test as implemented by Excel 2000 (Microsoft Corp., Redmond, WA). The mice were sacrificed 3 months after cell injection. Bone tumors were subjected to ex *vivo* imaging on a Lo-Rad M-IV mammography unit (Karmanos Cancer Institute, Detroit, MI) using a magnified specimen technique. Images were developed using a Kodak 2000 screen and radiography film (Kodak, Rochester, NY). Upon sacrifice, tumor tissue from each mouse was harvested and cut into two pieces; one part was frozen for molecular analysis, and the other part of the tissue was fixed in formalin followed by embedding in paraffin for histological evaluation and immunohistochemistry.

Tissue collection, fixation and H&E staining

Freshly harvested tumors grown in the implanted bones were fixed in 10% buffered formalin for 48 hours and decalcified with 10% EDTA, embedded and sectioned. Samples were then washed with tap water and soaked in a graded series of 50%, 60%, 70%, 80% and 90% ethanol for 30 minutes, and then in 90% and 100% ethanol for one hour. They were then held in a solution of 100 % ethanol and xylene at a 1:1 ratio for 30 minutes before being embedded in paraffin and held at 60°C for one hour to make paraffin blocks. Transverse sections (5 μm) were taken from the blocks and prepared for histochemical and immunohistochemical staining. Haematoxylin & Eosin (H&E) staining was used for histological observation.

Electrophoretic mobility shift assay for measuring NF-κB activity

Nuclear extracts were prepared from control and I3C-treated breast epithelial cells as previously described (4-6) and subjected to analysis for NF-κB DNA binding activity as measured by electrophoretic mobility shift assay. Using frozen tumor tissue, nuclear proteins were also extracted as previously described (4-6). Briefly, tissues were minced and incubated on ice for 30 min in 0.5 ml of ice-cold buffer A composed of 10 mM HEPES (pH 7.9), 1.5 mM KCl, 10 mM $MgCI_2$. 0.5 mM DTT, 10% NP-40, 0.1% IGEPAL CA-630 and 0.5 mM PMSF. The minced tissue was homogenized using a Dounce homogenizer (Kontes Co., Vineland, NJ) followed by centrifugation at 5000 x g at 4°C for 10 min. The supernatant (cytosolic proteins) was collected for Western Blot analysis and kept at -70°C until use. The crude nuclear pellet was suspended in 200 μl of buffer B (20 mM HEPES, pH 7.9; 25% glycerol, 1.5 mM $MgCl_2$; 420 mM NaCI: 0.5 mM DTT; 0.2 mM EDTA; 0.5 mM PMSF; and 4 μM leupeptidin) and incubated on ice for 30 min. The suspension was centrifuged at 16,000 x g at 4°C for 30 min. The supernatant (nuclear proteins) was collected and kept at -70°C until use. The protein concentration was determined using BCA protein assay (Pierce Chemical Co., Rockford, IL). Electrophoretic mobility shift assay was done by pre-incubating 8.0 μg of nuclear extract with a binding buffer containing 20% glycerol, 100 mM $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl, and 0.25 mg/mL poly (dI:dC) for 10 minutes. After the addition of IRDye-700 labeled NF-κB oligonucleotide, samples were incubated for an additional 20 minutes. The DNA-protein complexes were electrophoresed in an 8.0% native polyacrylamide gel and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1.

Statistical Analysis

The statistical significance was determined using student's *t*-test and $P<0.05$ was considered significant.

RESULTS

A. Development of a novel model of breast cancer grown in the environment of human bone:

This was the critical part of our project to develop an animal model of experimental breast cancer bone metastasis. But there are no other existing animal models in the literature that can be utilized faithfully for studying the breast cancer bone metastasis using breast cancer cell lines which can grow in the marrow of human bone implanted subcutaneously in to mice. This is the first report that we have established an animal model of experimental breast cancer bone metastasis using SCID-Hu animal **(Please see our preliminary data Fig. 1).**

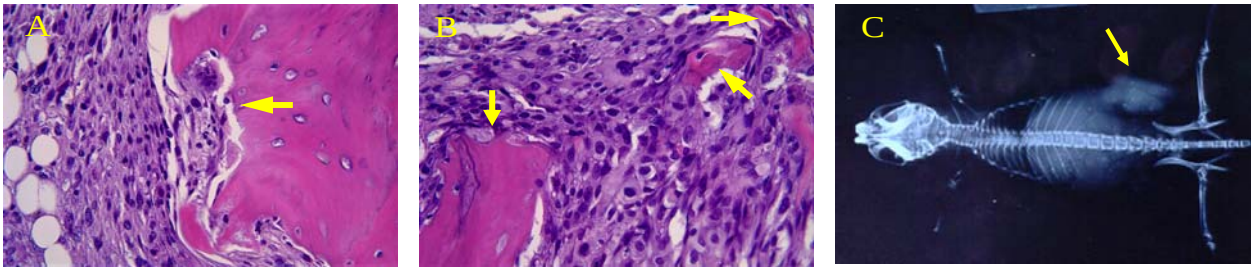

Fig. 1 *Typical osteolytic bone metastasis of MDA-MB-231 cells in SCID-Hu model. A: MDA-MB-231 cells which were injected into the implanted human fetal bone showing invasion (arrow); B: Continuous osteolytic and invasive process results in loss of the bone structure. Arrows indicate residuals bone surrounded by invasive tumor cells; C: The osteolytic bone metastasis of MDA-MB-231 cells, which eventually destroy fetal bone structure, as shown by X-ray examination.*

B. Effect of I3C during bone cancer metastasis:

Fig. 2 Schematic Design of Animal Experiment

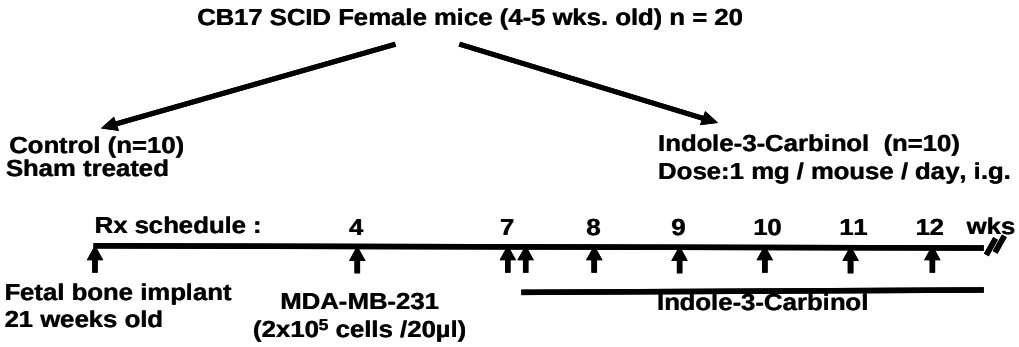

Fig 2: *Flow chart representation of in vivo experimental design and treatment schedule. The human fetal bone was implanted subcutaneously in the right flank of female homozygous CB17 SCID mice through a small skin incision with the open marrow cavity against the mouse muscle. Four weeks after the bone implantation, suspensions of MDA-MB-231 cells (2x10^5 cells) were injected through the mouse skin directly into the marrow of implanted fetal bone. The mice in the intervention group were given I3C (1*

mg/day/mouse) by oral gavage as soon as the majority of the bone implants began to enlarge (now called a "bone tumor") as determined by caliper measurements (23rd day after cancer cell injection). The control mice received only saline without I3C. The experiment was terminated 3 months after I3C treatment.

Inhibition of bone tumor growth by I3C

We utilized our recently established SCID-Hu model of experimental breast cancer bone metastasis to determine the effect of I3C *in vivo* in impeding tumor growth and metastasis. We found that I3C (1 mg I3C/day/mouse) significantly inhibited breast cancer bone tumor growth (Fig 3), documenting the efficacy of I3C in inhibiting breast cancer cell growth in the experimental model of bone metastasis. Under our experimental conditions, administration of I3C by gavage treatment caused 40% reduction in tumor volume (Fig 3), compared to control group. The statistical analysis indicated that compared to the

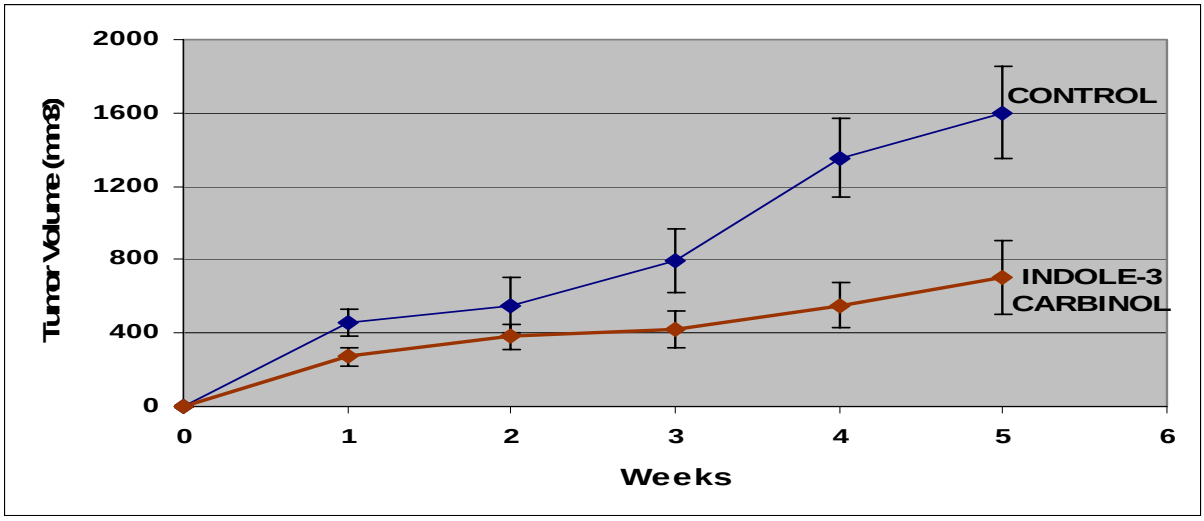

Fig. 3 *Inhibitory effects of I3C on the growth of xenografted bone tumors by MDA-MB-231cells in SCID-Hu mice. A total of 20 mice were divided into 2 groups. Under the experimental conditions, administration of I3C by gavage treatment caused 40% reduction in tumor volume, compared to control group.*

control group, bone tumor growth was significantly lower in the intervention (P = 0.05) group. At autopsy, all tumors were found localized at the site of injection with essentially spread to no other organs. Our treatment conditions did not cause any weight loss of the animals, suggesting that I3C did not induce any deleterious effects under the present experimental conditions. These results illustrate, for the first time, the efficacy of I3C in inhibiting tumor growth in an experimental breast cancer bone metastasis model.

Tumor histology and inhibition of osteolysis by I3C

H&E histology evaluation showed typical osteolytic bone metastasis of MDA-MB-231 cells in SCID-Hu animals (Fig 1). The osteolytic bone metastasis of MDA-MB-231 cells, which eventually destroyed fetal bone structure (arrow), was shown by X-ray examination (Fig 1). In

the control group, continuous osteolytic and invasive growth into the adjacent bone (arrow) resulted in gradual loss of the bone structure. Arrows indicate residual bone surrounded by invasive tumor cells (Fig 1).

Inhibition of NF-κB activation by I3C

To answer the most important question, whether treatment of animals with I3C could effectively target a specific signaling molecule such as NF-κB in tumor tissues, nuclear extract from frozen tumor tissues (randomly selected from control and treatment groups) was subjected to analysis for NF-κB DNA binding activity as measured by electrophoretic mobility shift assay. The results are shown in Fig 4, which clearly show that I3C was effective in down-regulating NF-κB in animal tumors receiving I3C compared to control tumors. These *in vivo* results were similar to our previously published data (6, 7), suggesting that the inactivation of NF-κB is, at least, one of the molecular mechanisms by which I3C induced anti-tumor activity in our experimental animal model. These data also provide proof of principle and suggest that I3C could be an effective anti-tumor agent in an animal model of breast cancer bone metastasis, which is mediated by inactivation of NF-κB DNA binding activity.

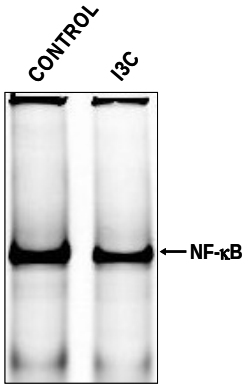

Fig. 4 *Nuclear extract (8 µg) was prepared from exponentially growing tumors in mice treated with I3C. Results indicate I3C was effective in down-regulating NF-κB in treated animals relative to control tumors.*

KEY RESEARCH ACCOMPLISHMENTS

- We have established an animal model of experimental human breast cancer bone metastasis.
- Determined the effects of I3C during the growth of MDA-MB-231 bone tumors in an animal model of human breast cancer metastasis.
- Determined the effect of I3C during breast cancer bone metastasis by down-regulating the expression of NF-κB in SCID-Hu animals.

REPORTABLE OUTCOMES

KM Wahidur Rahman, Sanjeev Banerjee, Joshua Liao and Fazlul H. Sarkar. The Role of SDF-1α and CXCR4 in Metastatic Breast Cancer, Proceedings of the Fourth Era of Hope, abstract # 60-21, 2005.

CONCLUSIONS

- MDA-MB-231 breast cancer cells grew in the human fetal bone and we have established an animal model of experimental breast cancer bone metastasis.
- I3C significantly inhibited the growth of MDA-MB-231 bone tumors in an animal model of human breast cancer metastasis.
- I3C inhibited breast cancer bone metastasis by down-regulating the expression of NF-κB in SCID-Hu animals.
- These results suggest that I3C could be a promising agent for the prevention and/or treatment of breast cancer and its metastasis.

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